

## Recombinant Technology

# Expression of single-chain Fv-Fc fusions in *Pichia pastoris*

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## Abstract

Phage display technology makes possible the direct isolation of monovalent single-chain Fv antibody fragments. For many applications, however, it is useful to restore Fc mediated antibody functions such as avidity, effector functions and a prolonged serum half-life. We have constructed vectors for the convenient, rapid expression of a single-chain antibody Fv domain (scFv) fused to the Fc portion of human IgG1 in the methylotrophic yeast *Pichia pastoris*. The scFv-Fc fusion protein is secreted and recovered from the culture medium as a disulfide-linked, glycosylated homodimer. The increased size of the dimer (~106 kDa vs. ~25 kDa for a scFv) results in a prolonged serum half-life in vivo, with  $t_{1/2}$  of the beta phase of clearance increasing from 3.5 h for a typical scFv to 93 h for a scFv-Fc fusion in mice. The scFv-Fc fusion is capable of mediating antibody-dependent cellular cytotoxicity against tumor target cells using human peripheral blood mononuclear cells as effectors. Finally, the Fc domain is a convenient, robust affinity handle for purification and immunochemical applications, eliminating the need for proteolytically sensitive epitope and/or affinity tags on the scFv. © 2001 Elsevier Science B.V. All rights reserved.

**Keywords:** *Pichia pastoris*; scFv; Antibody engineering; Phage display; HER2/neu; Botulism

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**Abbreviations:** Fv, antibody variable fragment; scFv, single chain Fv fragment; kDa, kilodaltons; V<sub>H</sub>, immunoglobulin heavy chain variable region; V<sub>L</sub>, immunoglobulin light chain variable region; C<sub>H</sub>2 and C<sub>H</sub>3, immunoglobulin heavy chain constant regions 2 and 3; ADCC, antibody-dependent cellular cytotoxicity; PCR, polymerase chain reaction; BoNT, botulinum neurotoxin; BoNT/A, BoNT type A; H<sub>c</sub>, BoNT binding domain; ELISA, enzyme linked immunosorbent assay; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; HRP, horse radish peroxidase; AMP, ampicillin; KAN, kanamycin; ZEO, Zeocin; PBS, phosphate buffered saline (25 mM NaH<sub>2</sub>PO<sub>4</sub>, 125 mM NaCl, pH 7.4); MPBS, 2% milk powder in PBS; FACS, fluorescence activated cell sorting; FITC, fluorescein isothiocyanate; PE, phycoerythrin; PBMC, peripheral blood mononuclear cells; RU, resonance units; ECD, extracellular domain; HBS, hepes buffered saline (10 mM Hepes, 150 mM NaCl, pH 7.4);  $k_{off}$ , dissociation rate constant;  $k_{on}$ , association rate constant;  $M_r$ , molecular weight; RT, room temperature; YNB, yeast nitrogen base (with ammonium sulfate without amino acids); i.v., intravenous; i.p., intraperitoneal.

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## 1. Introduction

The modular nature of antibody molecules allows for an almost unlimited number of domain rearrangements and fusions. Antibody engineering allows the researcher to design and use a variety of binding domains, effector domains, as well as non-antibody fusion partners (Chamow and Ashkenazi, 1999; Hoogenboom, 1997). One of the most useful antibody fragments is the single-chain Fv (scFv) in which the isolated antibody  $V_H$  and  $V_L$  domains are joined by a short peptide linker (Bird et al., 1988; Huston et al., 1988). This monovalent, minimal binding fragment is favored for antibody phage display techniques and phage antibody library construction (Marks et al., 1991; McCafferty et al., 1990).

Once a scFv has been isolated from a phage display library, the binding domain can be characterized with respect to affinity, epitope, and biological activity, as well as subjected to further affinity maturation. The scFv can be used as is, or engineered into other formats (Fv, Fab, (Fab')<sub>2</sub>, IgG, or fused to other proteins).

For some applications the scFv itself is the desired format. The small size of the scFv, its rapid clearance from the blood, and tumor penetration properties make it the format of choice for tumor targeting and radioimmunoimaging applications (Adams et al., 1995; Adams et al., 1998; Yokata et al., 1992). The single-chain nature of the scFv is also best-suited for intracellular immunization (intrabody) applications (Marasco, 1997). However for other uses, it would be desirable to transfer the antigen-binding properties of the scFv into a full length IgG, to take advantage of avidity effects, effector functions, and the prolonged serum half-life of an immunoglobulin. Increasing the serum half-life of scFv is particularly important for efficient in vivo antigen neutralization since the  $t_{1/2\beta}$  of a typical scFv is only 3.5 h in mice (Huston et al., 1996).

One approach is to directly engineer a phage displayed scFv into a full length IgG and express it in mammalian cells (Persic et al., 1997). However this approach requires separate cloning steps for the  $V_H$  and  $V_L$  domains, and establishing a stable antibody secreting cell line can be time consuming. An alternate approach would be to engineer the scFv

into a more 'IgG-like' structure that can be accomplished in a single cloning step, and that can be expressed at high levels in a eucaryotic microorganism such as yeast. We have constructed a vector to rapidly express an scFv-Fc fusion (wherein the scFv is fused to the hinge, C<sub>H</sub>2, and C<sub>H</sub>3 domains of human IgG1) in the methylotrophic yeast *Pichia pastoris*. The scFv-Fc fusion can easily be constructed in a single cloning step from a scFv and is secreted at high levels as a glycosylated dimer from *Pichia* cultures. The scFv-Fc fusion retains the affinity and specificity of the parent scFv, combined with the bivalency, prolonged serum half-life, and the Fc-mediated ADCC (antibody-dependent cellular cytotoxicity) of an IgG. These yeast-produced scFv-Fc fusions will be useful to rapidly characterize candidate scFv isolated from phage antibody libraries before converting to a full-length IgG, or as an alternative format in its own right.

## 2. Materials and methods

### 2.1. Cells and media

*Pichia pastoris* strain GS115 was obtained from Invitrogen, San Diego, CA. SKBR-3 and SK-OV-3 cells were obtained from the ATCC (American Type Culture Collection, Rockville, MD). Media used: YPD, yeast extract peptone dextrose medium (1% yeast extract, 2% peptone, 2% dextrose); YPDS, YPD medium plus 1 M sorbitol; BMGY, buffered glycerol complex medium (1% yeast extract, 2% peptone, 100 mM phosphate buffer pH 6.0, 1.34% YNB,  $4 \times 10^{-5}\%$  biotin, 1% glycerol); BMMY, buffered methanol complex medium (1% yeast extract, 2% peptone, 100 mM phosphate buffer pH 6.0, 1.34% YNB,  $4 \times 10^{-5}\%$  biotin, 0.5% methanol).

### 2.2. pIgG1, pIgG1-C25, and pIgG1-C6.5 plasmid construction

The Fc fragment (hinge, C<sub>H</sub>2 and C<sub>H</sub>3) of human IgG1 was amplified from the baculovirus expression plasmid pBHuCγ1 (Poul et al., 1995) using primers 'HuIgGNotIback' (5'-AAGGAAAAAGCGGCC-GCAGAGCCCCAAATCTTGTGACAAA-3') and 'HuIgGXbaIfor' (5'-ACGCTCTAGATCATTTACC-

CGGAGACAGGGAC-3'), which append *NotI* and *XbaI* sites (underlined) onto the 5' and 3' ends of the PCR fragment, respectively. The PCR fragment was digested with *NotI* and *XbaI* and subcloned into *NotI*-*XbaI* digested pPICZ $\alpha$ A (Invitrogen) to generate plasmid pPIgG1 (for 'Pichia IgG plasmid 1').

C25, a scFv which binds the botulinum neurotoxin type A binding domain (BoNT/A He) (Amersdorfer et al., 1997) was PCR amplified from vector pUC119MycHis-C25 using primers 'C25PichiaBack' (5'-CGGCAGCTCGAGAAAAGAGAGGCTGAAGCTCAGGTCCAGCTGCAGGAGTCTGGG-3') and 'LMB2' (5'-GTAAAACGACGGCCAGT-3'). The 5' PCR primer C25PichiaBack appends a *XhoI* site (underlined) onto the 5' end of the scFv for cloning into the *XhoI* site in the leader sequence of plasmid pPIgG1, and also recreates the last few amino acids of the leader sequence fused with the first codon of the C25 scFv. The 3' PCR primer LMB2 anneals outside of a unique *NotI* site at the end of the C25 scFv, derived from plasmid pHEN1 (Hoogenboom et al., 1991). The PCR fragment was then digested with *XhoI* and *NotI* and subcloned into *XhoI* and *NotI* digested plasmid pPIgG1 generating plasmid pPIgG1-C25. Similarly, C6.5, a scFv which binds the extracellular domain of the HER2/*neu* oncoprotein (Schier et al., 1995) was subcloned into pPIgG1 using primers 'C6.5PichiaBack' (5'-CGGCAGCKC-GAGAAAAGAGAGGCTGAAGCTGGCCAGGTG-CAGCTGGTGCAG-3') and 'LMB2' to create plasmid pPIgG1-C6.5. Following construction, the coding sequences for the Fc region and both the scFvs were sequenced to exclude any PCR-induced errors.

### 2.3. Electroporation

*Pichia pastoris* strain GS115 was transformed by electroporation. 10  $\mu$ g of plasmid was linearized with *PmeI*, phenol-chloroform extracted, ethanol precipitated, and dissolved in 10  $\mu$ l of dH<sub>2</sub>O. Preparation of electrocompetent *Pichia* strain GS115 was done as per supplier's instructions (Invitrogen). 80  $\mu$ l of electrocompetent cells were mixed with 5–10  $\mu$ g of linearized plasmid in a 0.2 cm electroporation cuvette, incubated on ice 5 min, and electroporated in a Biorad GenePulser with settings of 1500 V, 25  $\mu$ F capacitance, and 400 ohms resistance. After pulsing, 1.0 ml of ice cold 1 M sorbitol was added

immediately to the cuvette, and the cells transferred to a sterile 15 ml culture tube. The tube was incubated at 30°C without shaking for 1 h, then 1.0 ml YPD medium was added to the tube, and the cells were allowed to recover for 2 h at 30°C at 250 RPM. Transformants were plated (200  $\mu$ l) on YPDS plates containing 100  $\mu$ g/ml Zeocin and grown at 30°C to isolate Zeocin-resistant transformants.

### 2.4. Small scale expression and screening of *Pichia* transformants

Zeocin-resistant transformants of pPIgG1-C25, pPIgG1-C6.5 and pPICZ $\alpha$ A (control) plasmids were grown overnight in BMGY medium at 30°C and 250 rpm shaking in 100 ml glass culture tubes. The next day, the cells were recovered by centrifugation and resuspended to an OD<sub>600</sub> of 1.0 in BMMY media to induce, and grown again at 30°C and 250 rpm. Fresh methanol was added to a total of 0.5% to maintain induction at 24, 48 and 72 h post induction. After 72 h, samples of the media were analyzed by SDS-PAGE followed by silver stain (Biorad Silver Stain Plus, Biorad, Hercules, CA) and Western blot with polyclonal goat anti-human IgG (Fc-specific) conjugated to horse radish peroxidase (A-0170, Sigma, St. Louis, MO). Detection was with the chemiluminescent substrate ECL (Amersham Pharmacia Biotech, Piscataway, New Jersey).

### 2.5. Large scale expression and purification of scFv-Fc fusions

250 ml cultures of GS115/pPIgG1-C25 and GS115/pPIgG1-C6.5 were grown overnight in BMGY plus Zeocin (50  $\mu$ g/ml) and kanamycin (50  $\mu$ g/ml) until the OD<sub>600</sub> was 4–6. The cells were recovered by centrifugation, then diluted to an OD<sub>600</sub> of 1.0 in 1000 ml of fresh BMMY media containing 50  $\mu$ g/ml kanamycin to induce. The 1000 ml of resuspended cells were divided equally between 4  $\times$  2 l baffled-bottom flasks to ensure adequate aeration, and grown at 30°C and 250 rpm. Fresh methanol was added to 0.5% to maintain induction at 24, 48, and 72 h. After 72 h, the cells were removed by centrifugation and the scFv-Fc fusion protein purified from the supernatant. Twenty five milliliters of a protease inhibitor cocktail (P2714, Sigma) was added

to reduce proteolysis, and the pH of the supernatant was adjusted to 8.0 by the addition of 1/10 volume of 1.0 M Tris pH 8.0. Proteins were precipitated by the addition of 400 g ammonium sulfate in the cold with constant stirring over a period of 2 h. The precipitate was recovered by centrifugation, dissolved in 40 ml of 25 mM Tris pH 8.0, and dialyzed overnight against two changes of 4 l apiece of 25 mM Tris pH 8.0 at 4°C. After dialysis, the sample was applied to a 1.5 ml Protein G column (Sigma) that had been previously equilibrated with 25 mM Tris pH 8.0. The column was washed once with 10 ml of 100 mM Tris pH 8.0, once with 10 ml of 10 mM Tris pH 8.0, and eluted with 20 ml of 100 mM glycine pH 3.0. 1.0 ml fractions were collected in Eppendorf tubes containing 100 µl of 1.0 M Tris pH 8.0 to neutralize. Peak fractions were determined by absorbance at 280 nm, pooled, concentrated to 0.5 ml on a Centricon 10 concentrator (Amicon, Beverly, MA) and finally gel filtered on a Superdex S-200 column (Amersham Pharmacia Biotech) at a flow rate of 0.5 ml per min in PBS. Protein concentrations were determined based on absorbance at 280 nm using molar extinction coefficients calculated by the method of Gill and von Hippel (Gill and Hippel, 1989). We derived a factor of 1.0  $A_{280}$  is equal to 540 µg/ml for C6.5-Fc and 620 µg/ml for C25-Fc, based on a calculated molecular weight of 106,000 for each.

## 2.6. Glycosylation analysis

Samples of the scFv-Fc fusions were de-glycosylated using PNGase F (New England Biolabs, Beverly, MA) under denaturing according to the manufacturer's instructions and analyzed by SDS-PAGE followed by silver staining (Biorad Silver Stain Plus).

## 2.7. Cytotoxicity assay

Procedures were performed in round-bottom microtiter plates (Costar) using a lactate dehydrogenase (LDH) detection kit (Boehringer Mannheim). Briefly, 10,000 HER2/*neu* expressing target cells (SKBR-3) in 50 µl of complete media/well were co-cultured with various numbers of effector peripheral blood mononuclear cells (PBMC) added in 100 µl medium/well. Different concentrations of C6.5-Fc

fusion protein antibody were added to appropriate cultures in 50 µl volumes (final volume per well, 200 µl). Cultures were performed in triplicates and plates were incubated for 4 h, after which time the plates were centrifuged and 100 µl supernatant were carefully removed from each well and transferred to corresponding wells of a flat bottom microtiter plate. To determine the LDH activity, 100 µl reaction mixture (cytotoxicity kit) are added to each well and the plates were further incubated for 30 min at room temperature for color development. The amount of LDH activity released and detected colorimetrically in the culture supernatant correlates to the proportion of lysed cells. The absorbance of the supernatants is measured at 490 nm and the data are expressed as mean absorbance. The percent cytotoxicity was calculated as follows: percent cytotoxicity =  $[(A - B)/(C - B)] \times 100$  where  $A$  = the mean absorbance of supernatants from test cultures;  $B$  = the mean absorbance of supernatants from cultures containing target cells only (spontaneous LDH release); and  $C$  = the mean absorbance of supernatants from cultures containing target cells cultured in the presence of 2% Triton (maximum LDH release). The data were adjusted in accordance with absorbance of supernatants from cultures of PBMC plus C6.5-Fc fusion protein as well as the absorbance of medium devoid of any cells.

## 2.8. Equilibrium constant ( $K_d$ ) determination

Affinity measurements were performed on a Kinexa instrument from Sapidyn Instruments, Boise, ID. PMMA beads (Sapidyn Instruments) were coated with antigen (either recombinant BotNT/A Hc fragment or HER2/*neu* extracellular domain) at 50 µg/ml in PBS overnight at 4°C. The beads were washed three times with PBS and blocked with 0.2 µm filtered 2% skimmed milk powder in PBS (MPBS) for 30 min. scFv-Fc fusions were incubated with dilutions of antigen for 4 h in MPBS. The concentration of the C6.5-Fc fusion in the reaction was 10 nM and 1 nM for the C25-Fc fusion. The amount of uncomplexed antibody in the equilibrium reaction was quantified by capture on the antigen coated PMMA beads, followed by detection with 1:1000 dilution of Cy5 labeled goat anti-human Fc antibody (Jackson Laboratories). Reactions were run in duplicate and fluorescent signals were plotted

as function of antigen concentration. Equilibrium constants were determined using the software provided by the manufacturer (Sapidyne Instruments). The Kinexa assay has been described in detail (Blake et al., 1997).

### 2.9. Radiolabeling

The C6.5-Fc was labeled with iodine-125 using iodobeads (# 28665X, Pierce; Rockford IL) according to the methods described by the manufacturer. Briefly, 0.5 mg of C6.5-Fc in 250 ml 0.1 M phosphate buffer (pH 6.5) were combined with 0.5 mCi (1.25 ml) of iodine-125 (#NEZ033H, DuPont NEN, Wilmington, DE), and one iodobead. The mixture was incubated for 3 min at room temperature. Unincorporated radioiodine was separated from the labeled protein by gel filtration using the G-50-80 centrifuged-column method (Meares et al., 1984). The immunoreactivity of the C6.5-Fc was evaluated in a live cell binding assay utilizing HER2/*neu* expressing SK-OV-3 cells (Adams et al., 1993). Ten nanograms of labeled C6.5-Fc in 100 ml PBS was added in triplicate to  $5 \times 10^6$  SK-OV-3 cells in 15 ml polypropylene centrifuge tubes. After a 30 min incubation at room temperature the cells were washed with 2.0 ml of PBS and centrifuged for 5 min at  $500 \times g$ . Supernatants were separated from the cell pellets, both were transferred to  $12 \times 75$  counting tubes, counted in a gamma well counter (Gamma 4000, Beckman Instruments, Irvine, CA) and the percentage of activity associated with the cell pellet was determined. Fifty three percent of the activity was found to be associated with the cell pellet. While the maximum possible value in this assay is about 80%, the degree of retention is dictated by a number of factors including the affinity of the molecule for the target antigen. These results are within the normal range observed for radioiodinated C6.5 scFv, indicating to us that the C6.5-Fc was still reactive with cell surface HER2/*neu*.

### 2.10. Pharmacokinetics

Four-month-old inbred male C.B17/Icr-*scid* (*scid*) mice were obtained from the Fox Chase Cancer Center Laboratory Animal Facility. Lugol's solution was placed in the drinking water to block thyroid accumulation of radioiodine. Two days later, phar-

macokinetic studies were initiated. Twenty micrograms of  $^{125}\text{I}$ -C6.5-Fc were administered to three cohorts of four mice by i.v. tail vein injection and to three cohorts of four mice by i.p. injection. Total injected doses were determined by counting each animal on a Series 30 multichannel analyzer/probe system (probe model #2007, Canaberra, Meridian, CT). Blood samples (20–75  $\mu\text{l}$ ) were obtained by retro-orbital bleeds from all mice at 5 min post injection and then from alternating groups at subsequent time points (e.g., group I at 15 min, group II at 30 min, group III at 1 h, etc.). The blood samples were counted along with standards in a gamma counter (Beckman, 4000) and the percent of the injected dose retained per ml (% ID/ml) of blood over time was determined for each mouse. The mean values were determined for each time point and the pharmacokinetics were determined using the NCOMP program (Laub and Gallo, 1996).

## 3. Results

### 3.1. Plasmid constructions

We have constructed a plasmid for the expression of single-chain antibody Fv domains (scFv) fused to the human IgG1 Fc domain to make a bivalent,  $\sim 106,000$  molecular weight scFv-Fc fusion (Fig. 1). Plasmid pPIgG1 for the expression of the scFv-Fc fusions (comprising the scFv followed by the hinge,  $\text{C}_{\text{H}}2$  and  $\text{C}_{\text{H}}3$  domains of human IgG1) is based on the *Pichia pastoris* expression plasmid pPICZ $\alpha$ A (Invitrogen). This plasmid uses the alcohol oxidase (AOX1) promoter for high level expression of heterologous proteins, which are secreted to the media under direction of the *Saccharomyces cerevisiae* a-factor signal sequence.

Details of the plasmid and the cloning sites are shown in Fig. 2. The human hinge,  $\text{C}_{\text{H}}2$ , and  $\text{C}_{\text{H}}3$  domains of IgG1 were cloned into the *Not*I and *Xba*I sites of pPICZ $\alpha$ A, creating plasmid pPIgG1. A stop codon was included in the construct following the final amino acid of the  $\text{C}_{\text{H}}3$  domain; therefore the myc epitope tag and  $(\text{His})_6$  sequences from pPICZ $\alpha$ A are not expressed in the fusion protein.

scFv genes to be expressed as scFv-Fc fusions in pPIgG1 are amplified by PCR and subcloned into pPIgG1 using the 5' *Xho*I site in the alpha factor

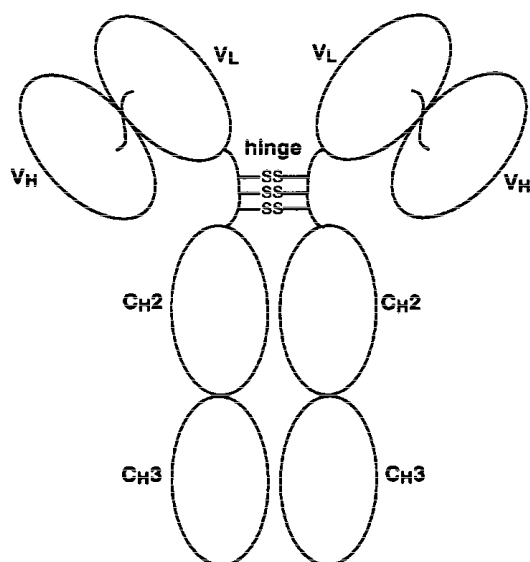


Fig. 1. Schematic representation of a dimeric scFv-Fc fusion.  $V_H$ , heavy chain variable domain;  $V_L$ , light chain variable domain;  $C_{H2}$  and  $C_{H3}$ , heavy chain constant domains 2 and 3; –SS– indicates disulfide bonds between the hinge domains.

signal sequence and the 3' *NotI* site which sits just before the beginning of the IgG hinge (Fig. 2). The 5' PCR primer, in addition to appending an *XhoI*

site, must also be designed to recreate the amino acid sequence of the alpha amylase signal from the *XhoI* site to the end of the signal peptide, positioning the 5' end of the scFv flush with the end of the signal. The 3' PCR primer can be either a sequence-specific primer which appends a *NotI* site onto the end of the scFv gene, or a universal downstream primer for amplifying from vectors such as the phage display vector pHEN1 which already has an in-frame *NotI* site positioned at the end of the scFv gene (Hoogenboom et al., 1991). We subcloned two model scFvs into the pPIgG1 vector for evaluation: C25, which recognizes the botulinum neurotoxin serotype A binding domain (BotNT/A Hc) (Amersdorfer et al., 1997), and C6.5, which recognizes the HER2/*neu* protein (Schier et al., 1995). The constructs were electroporated into *Pichia pastoris* strain GS115 for expression.

### 3.2. Small scale expression and screening of *Pichia* transformants

Since protein expression levels in *Pichia* from integrated pPICZ $\alpha$ -derived plasmids can vary depending on site of integration and copy number, we

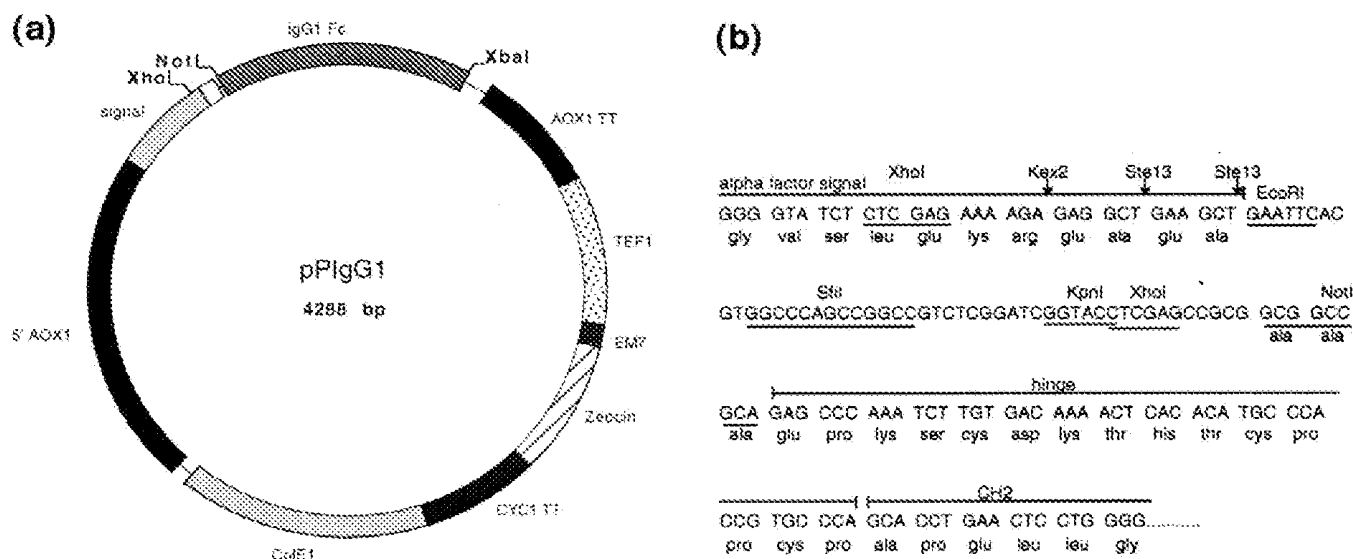


Fig. 2. (A) Schematic of plasmid pPIgG1. 5' AOX1, alcohol oxidase 1 promoter; AOX1 TT, transcriptional terminator from *Pichia pastoris* AOX1 gene; TEF1 promoter, transcriptional elongation factor 1 promoter from *Saccharomyces cerevisiae*; EM7 promoter, synthetic prokaryotic promoter; Zeocin, Zeocin resistance gene; CYC1 TT, transcriptional terminator from *Saccharomyces cerevisiae* CYC1 gene; ColE1, ColE1 origin of replication. (B) Details of the cloning sites of pPIgG1. Restriction endonuclease sites are underlined, arrows indicate cleavage sites within the alpha mating factor signal sequence for Kex2 and Ste13 proteases.

screened multiple transformants for scFv-Fc expression levels. Four clones apiece of the pPIgG1-C25 and pPIgG1-C6.5 Zeocin-resistant transformants (along with pPICZ $\alpha$ A as a control) were grown and induced in 10 ml of BMMY media at 30°C for 72 h as described in Materials and methods. Cultures supernatants were analyzed on SDS–PAGE under reducing conditions followed by silver stain detection. Results show that a new band appears in the media with an apparent molecular weight of ~55,000 daltons, in good agreement with the predicted 53,000 dalton molecular weight of a reduced scFv-Fc fusion (Fig. 3 panel A). Close inspection shows that this band is actually a closely spaced doublet of bands with similar mobilities. Western blotting analysis demonstrates that these bands react with an anti-human Fc specific antibody (Fig. 3 panel B). No anti-Fc reactive species were seen in *Pichia* GS115 cells transfected with the control plasmid

(pPICZ $\alpha$ A). On non-reducing SDS–PAGE gels, the scFv-Fc protein bands have approximately twice the apparent molecular weight, indicating that the scFv-Fc fusions are secreted to the media primarily as disulfide-linked dimers (not shown). Expression levels of the C6.5-Fc clones are somewhat less than that of the C25-Fc clones. Two well-expressing clones were chosen for large scale expression and analysis.

### 3.3. Large scale expression and purification of scFv-Fc fusions

One liter of culture media was used to generate pure C25-Fc and C6.5-Fc fusions for characterization. Briefly, the purification employed was ammonium sulfate precipitation of the proteins from the culture supernatants, followed by dialysis, Protein G affinity chromatography, and gel filtration chroma-

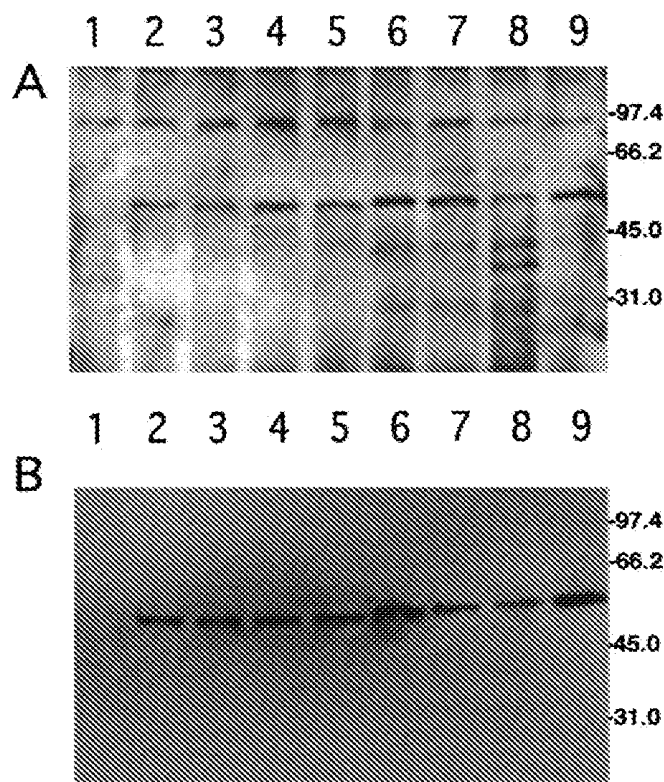


Fig. 3. Analysis of scFv-Fc expression in *Pichia pastoris* strain GS115. (A) Silver stained SDS–PAGE gel of culture supernatants. Lane 1, pPICZ $\alpha$ A control transformant; lanes 2–5, pPIgG1-C6.5 transformants; lanes 6–9, pPIgG1-C25 transformants. (B) Western blot of a duplicate of the above gel using anti-human IgG (Fc-specific)-HRP (horse radish peroxidase) conjugate, followed by ECL development. Molecular weight markers (kDa) are indicated on right of each gel.

tography. Fig. 4, panel A shows fractions from a typical purification. After ammonium sulfate precipitation, the ~55 kDa scFv-Fc fusion protein doublet is clearly visible (lanes 1 and 4). After Protein G

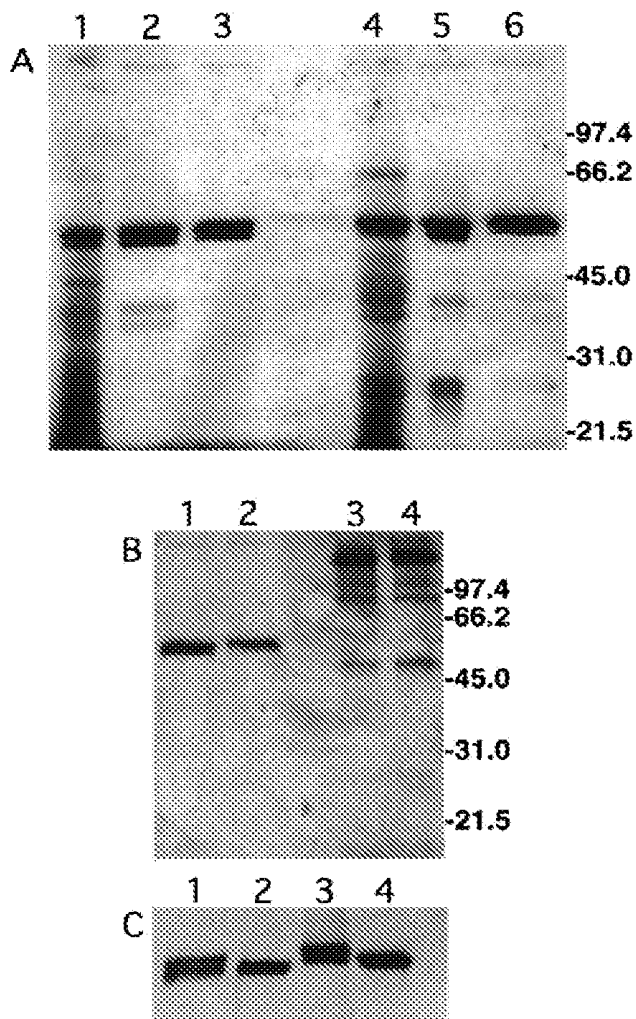


Fig. 4. (A) SDS-PAGE analysis of samples from representative purifications of the C6.5 and C25-Fc fusion proteins. Lane 1, ammonium sulfate pellet of C6.5-Fc; lane 2, protein G pool of C6.5-Fc; lane 3, S200 pool of C6.5-Fc; lane 4, ammonium sulfate pellet of C25-Fc; lane 5, protein G pool of C25-Fc; lane 6, S200 pool of C25-Fc. (B) SDS-PAGE analysis of the purified scFv-Fc fusions under reducing and non-reducing conditions. Lanes 1 and 3, purified C6.5-Fc, lanes 2 and 4, purified C25-Fc protein. Lanes 1 and 2 are under reducing conditions, lanes 3 and 4 under non-reducing conditions. Molecular weight standards (kDa) are to the right. (C) PNGase F analysis of glycosylation of the C6.5-Fc and C25-Fc fusions. Lane 1 and 2, C6.5-Fc; lanes 3 and 4, C25-Fc. Lanes 1 and 3 are minus PNGase F; lanes 2 and 4 are plus PNGase F.

affinity chromatography (lanes 2 and 5) the scFv-Fc fusion protein is substantially pure, with a number of smaller bands copurifying which may represent breakdown products which also bind to Protein G. These lower molecular weight species are removed by gel filtration on Superdex S-200 (Amersham Pharmacia Biotech) (lanes 3 and 6). The yield after purification was ~2 mg/l of culture for C25-Fc and ~300 µg/l of culture for the C6.5-Fc fusion. The purified scFv-Fc fusion protein is a disulfide-linked dimer, as shown by SDS-PAGE analysis under reducing and non-reducing conditions (Fig. 4B). The dimeric nature of the scFv-Fc was also confirmed by molecular weight analysis on Superdex S-200 gel filtration vs. molecular weight standards (not shown).

The final purified scFv-Fc proteins migrate in reducing SDS-PAGE as a tight doublet of bands with an apparent molecular weight of ~55,000 daltons. The molecular weight heterogeneity observed could be due to differences in glycosylation, or in the processing of the alpha amylase signal peptide. To explore these possibilities, the purified fusions were subjected to N-terminal sequencing and de-glycosylation analysis with PNGase F.

N-terminal analysis results indicated that both the upper and lower bands of the doublet, for both C6.5-Fc and C25-Fc proteins, begin with the amino acid sequence glu-ala-glu-ala..., indicating that signal peptide cleavage had occurred after the Kex2 site in the alpha amylase leader, but that Ste13 cleavage had not occurred (Fig. 2). The samples were further analyzed by deglycosylation with PNGase F (Fig. 4C). After deglycosylation of both the fusion proteins, the doublet collapsed to a single band, indicating that the observed heterogeneity is due to N-linked glycosylation of the higher molecular band and no glycosylation of the lower molecular weight band.

### 3.4. Affinity ( $K_d$ ) analysis

We wished to show that the scFv antigen binding site in the scFv-Fc fusion retained the affinity of the unfused scFv. The affinities of our model scFvs for their antigens have been previously determined by kinetic analysis of association and dissociation rate constants ( $k_{on}$  and  $k_{off}$ ) by surface plasmon resonance (Amersdorfer et al., 1997; Schier et al., 1995);



from the  $k_{on}$  and  $k_{off}$  values the equilibrium dissociation constants ( $K_d$ s) of C6.5 and C25 were calculated to be 16 nM and 1 nM, respectively. We determined equilibrium dissociation constants under equilibrium conditions using a Kinexa instrument (Sapidine Instruments) (Blake et al., 1997). We determined the  $K_d$ s of the C6.5-Fc and C25-Fc fusion proteins to be 4.6 nM and 0.5 nM, respectively, for their cognate antigens. While the different experimental techniques do not allow a direct comparison of the  $K_d$  values, and we cannot rule out subtle differences in affinity between the scFv and scFv-Fc fusion formats, it is clear that the scFv binding sites retain high affinity for their cognate antigens in the scFv-Fc fusion format.

### 3.5. ADCC assays

The C6.5-Fc fusion protein was assayed to determine if the Fc domain of the fusion was functionally able to direct antibody-dependent cell cytotoxicity (ADCC) towards antigen expressing target cells. 10,000 HER2/*neu* expressing SKBR3 target cells were co-cultured with effector PBMC (peripheral blood mononuclear cells) at 100:1, 50:1, and 25:1 ratios in the presence of various concentrations of C6.5-Fc. After 4 h incubation, the samples were analyzed for percent cell lysis by lactate dehydrogenase (LDH) release. Results (Fig. 5) show PBMC killing of target cells occurs in a dose dependent fashion with C6.5-Fc.

### 3.6. Pharmacokinetics

Single-chain Fv antibodies are rapidly cleared from the bloodstream in mouse models, with typical  $t_{1/2}$  of the beta clearance of approximately 3.5 h (Huston et al., 1996). Similarly, in previous studies of C6.5 scFv pharmacokinetics in scid mice, a  $t_{1/2}$   $\beta$  of 3.0 h was determined (G. Adams, unpublished data). This rapid clearance prevents the characterization of scFv in animal models for efficacy where a longer serum half-life is required to observe a biological effect. We wished to determine if the fusion of a model single-chain antibody Fv domain to a Fc domain would impart a longer serum half life to the scFv. C6.5-Fc was labeled with iodine-125 using iodobeads, evaluated in a live cell binding

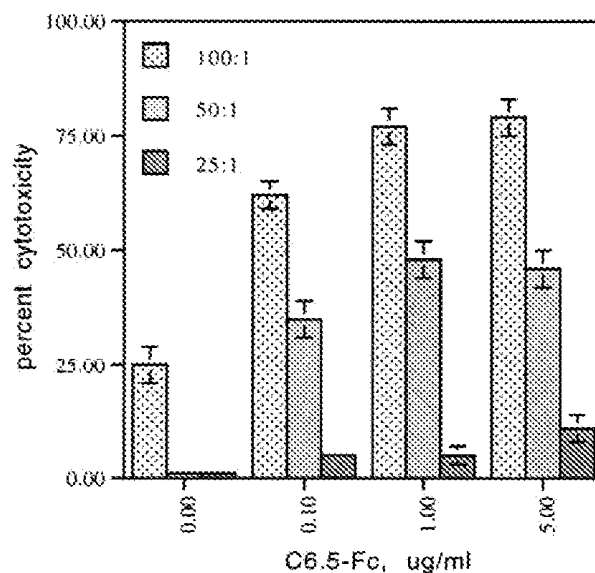


Fig. 5. ADCC (antibody-dependent cellular cytotoxicity) assays of the C6.5-Fc fusion protein using PBMC effector cells and HER2/*neu* expressing SKBR3 cells as target. Shaded bars indicates different effector cell: target cell ratios.

assay utilizing HER2/*neu* expressing SK-OV-3 cells for retention of activity. (Adams et al., 1993), and administered to four month old inbred male C.B17/Icr-*scid* (*scid*) mice as described in Materials and methods. Both intravenous (tail vein) and intraperitoneal routes of administration were studied. Results are shown in Fig. 6. After a rapid tissue distribution (alpha phase), the scFv-Fc fusion is cleared in the beta phase of elimination more slowly, with the  $t_{1/2}$   $\beta$  of 37.3 h for intravenous and 92.8 h for intraperitoneal injections. This represents a more than 12-fold and more than 30-fold improvement over the 3.0 h  $t_{1/2}$   $\beta$  observed for a C6.5 scFv alone. In contrast to a typical scFv, which is practically undetectable in mouse serum 12 h past injection, the slower clearance of the scFv-Fc fusion results in substantial levels of scFv in the blood at longer times, with approximately 5% of the injected dose still in the serum at 48 h after injection for both i.v. and i.p. injections.

## 4. Discussion

The single-polypeptide, minimally sized scFv antibody fragment is useful for the design and

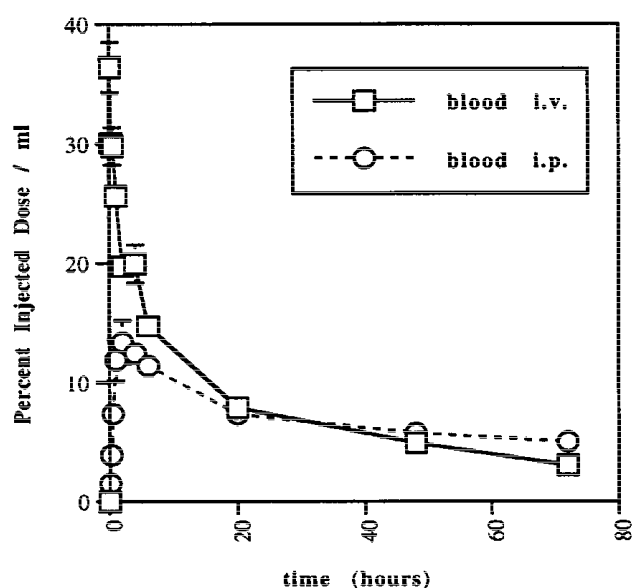


Fig. 6. Kinetics of clearance of C6.5-Fc fusion from mouse serum. Mice received  $^{125}$ I-labeled C6.5-Fc protein by either intravenous or intraperitoneal injection. Each data point represents the mean value for four mice.

construction of phage display antibody libraries, as fusion proteins, and as intrabodies; in addition the small size is best suited to tumor targeting due to superior tissue penetration (Yokata et al., 1992). However useful, the scFv format suffers from a number of key limitations. The lack of avidity due to monovalent binding can limit the effectiveness and/or sensitivity of scFv fragments in many immunochemical applications such as FACS and ELISA. Also, the scFv fragment typically needs to be 'tagged' in some way, such as a hexahistidine or epitope tag, to allow detection and purification. Perhaps most importantly, in vivo characterization of scFv in animal models can be impossible because of their rapid clearance from the bloodstream due to their small size. (Huston et al., 1996)

It would be desirable to be able to easily reengineer a scFv into an IgG-like format that combines the affinity and specificity of the scFv with the bivalency, pharmacokinetics, and effector functions of a complete immunoglobulin. One way is to clone the  $V_H$  and  $V_L$  genes from the scFv and reclone them into a full-length IgG expressing vector (Persic et al., 1997). However this requires separate cloning steps for the  $V_H$  and  $V_L$ , and it can require significant

amounts of time to make a stable IgG secreting tissue culture line.

As an alternative, we have constructed a vector, pPIgG1, to express a scFv-Fc fusion (in which the scFv is fused to the hinge,  $C_H2$ , and  $C_H3$  domains of human IgG1) in the yeast *Pichia pastoris* (Fig. 1). The scFv is expressed and secreted as a glycosylated, disulfide-linked dimer at high yields in the culture medium. The scFv-Fc fusion combines the affinity and specificity of the scFv antigen binding site with Fc-mediated dimerization. In addition, the Fc domain is a convenient affinity 'handle' for the purification and detection of the scFv species by reagents like protein A, protein G, and anti-Fc antibodies (Figs. 3B and 4A). An additional major advantage of *Pichia pastoris* over mammalian cell culture production is that *Pichia* can be readily fermented to high OD and high protein expression levels (Cregg and Higgins, 1995). This construct is similar to scFv-Fc fusions that other workers have made in mammalian and insect cells (Brocks et al., 1997; Hayden et al., 1994; Kato et al., 1995; Ma et al., 1996; Shu et al., 1993), however this is the first time to our knowledge that such a fusion has been expressed in yeast.

We have cloned and expressed two model scFv into pPIgG1 for evaluation of our system: C25, which recognizes the botulinum neurotoxin serotype A binding domain (BotNT/A Hc) (Amersdorfer et al., 1997), and C6.5, which recognizes the HER2/*neu* protein (Schier et al., 1995). Yields of the scFv-Fc fusions vary depending on the nature of the scFv, from ~2 mg/l for the best expressor (C25-Fc) to ~300  $\mu$ g/l for the C6.5-Fc fusion in shaker flasks. By an equilibrium  $K_d$  determination, we have shown that the scFv domain of the fusion retains the affinity of the parent scFv for its cognate antigen.

The use of scFv antibody fragments in FACS can be limited due to monovalent binding and lack of a good detection method. In favorable circumstances, we and other investigators have demonstrated the use of scFv from phage display libraries as FACS reagents, either directly as scFv-phage, or as isolated scFv fragments (de Kruif et al., 1995). However, we have found that the utility of scFv as FACS reagents must be evaluated on a case-by-case basis. For example, previous studies of C6.5 scFv protein on HER2/*neu* positive SK-OV-3 cells showed that the

half-life ( $t_{1/2}$ ) of the monovalent C6.5 scFv on the cell surface was much less than 5 min; efficient staining required preparing biotinylated scFv, staining the cells with biotinylated scFv, fixing the stained cells in paraformaldehyde, and detection with PE labeled streptavidin (Schier et al., 1996). In contrast, we routinely use the C6.5-Fc fusion protein in FACS assays on HER2/*neu* expressing cells without any modification of the protein or fixing of the target cells; detection is accomplished with fluorescently labeled anti-Fc antibodies (M.A. Poul, not shown). In addition, superior binding of a bivalent scFv-Fc to a cell surface antigen due to avidity also results in superior sensitivity over a monovalent scFv. This could be of obvious benefit for use of phage display library derived scFv to study cell surface expression of target molecules, especially where low levels of cell surface antigen need to be detected.

For in vivo characterization of scFv species, a long serum half-life will be needed in many instances to observe a biological effect. Previous experiments in mice have shown that the  $t_{1/2}$  for the beta phase of elimination for scFvs are typically 3.5 h (Huston et al., 1996). Similarly, in previous studies of C6.5 scFv pharmacokinetics in scid mice, a  $t_{1/2}$   $\beta$  of 3.0 h was determined (G. Adams, unpublished data). We have shown in these experiment a significant increase of the  $t_{1/2}$   $\beta$  of the C6.5-Fc fusion to 37.3 and 92.8 h for intravenous and intraperitoneal injections, respectively. This represents greater than 12-fold and 30-fold improvements in the half life, resulting in significant serum levels (~5% of the injected dose/ml) even at 48 h post injection. We have recently used the superior pharmacokinetics of pPIgG1 derived scFv-Fc fusions to demonstrate in vivo Botulinum toxin neutralization with scFv selected from phage display libraries (Amersdorfer, Powers, and Marks, manuscript in preparation).

In contrast to a typical immunoglobulin, pharmacokinetic analysis of the C6.5-Fc fusion when administered by i.v. injection shows a significant and rapid clearance of ~80% of the fusion protein in the alpha phase (Fig. 6). We speculate that this may be due to the nature of carbohydrate attached to the *Pichia* derived scFv-Fc fusion. Glycosylation patterns on immunoglobulin species can have profound effects on antibody effector function and phar-

macokinetics (reviewed in Wright and Morrison, 1997). Yeast in general attach high-molecular weight mannose structures (Jahn-Schmid et al., 1996), although in *Pichia pastoris* these residues are generally shorter than in *Saccharomyces cerevisiae* (Cregg and Higgins, 1995). Terminally mannosylated carbohydrates on immunoglobulins can be rapidly cleared by binding to high-affinity mannose receptors in the liver; this clearance can be partially prevented by co-injection of mannan (Wright and Morrison, 1994). Since complete deglycosylation of an IgG results in normal antigen affinity while retaining some (but not all) effector functions (Leatherbarrow et al., 1985; Tao and Morrison, 1989; Walker et al., 1989; Wright and Morrison, 1997), it may be possible to further improve the pharmacokinetics of the *Pichia* derived scFv-Fc fusions by eliminating the N-linked glycosylation site from the Fc domain by site-directed mutagenesis.

We have recently modified the pPIgG1 vector to further increase its utility. At present cloning into pPIgG1 requires using the 5' *Xho*I site in the alpha factor signal sequence and reconstruction by the PCR primer of the last few amino acids in the signal sequence. Since N-terminal sequence analysis shows that the final four amino acids of the signal (glu-ala-glu-ala...) are not cleaved from the C6.5-Fc and C25-Fc fusions, we have mutated the alpha factor signal sequence to end in glu-ala-met-ala..., and incorporated a *Nco*I site (CCATGG) around the minus 2 position methionine codon (ATG). In addition a second *Nco*I site in the pPICZ $\alpha$ A vector was eliminated by site-directed mutagenesis. This modified vector (pPIgG2) allows scFv to be removed and subcloned by a simple *Nco*I-*Not*I digest from our preferred scFv phage display vector pHEN1 (Hoogenboom et al., 1991) (D. Powers, unpublished results).

As more and more scFv from phage display libraries are developed for research, diagnostic, and therapeutic uses, it will be increasingly more important to have simple, rapid techniques to reengineer the scFv into appropriate formats for characterization. The *Pichia pastoris* scFv-Fc fusion protein system allows a simple, rapid way to combine the affinity and specificity of a scFv with the Fc mediated bivalency, prolonged serum half life, and effector functions of an IgG.

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